

# INHIBITION OF AEROBIC AND ANAEROBIC GROWTH OF STAPHYLOCOCCUS AUREUS IN A MODEL SAUSAGE SYSTEM

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## ABSTRACT

*Sodium nitrite, potassium sorbate, and glycerol monolaurate all inhibited anaerobic growth of Staphylococcus aureus more than its aerobic growth in an agar-meat model sausage system, and all were more inhibitory when lactic acid was added. Whereas anaerobic growth of S. aureus was inhibited by concentrations of 100, 2500, and 2500 ppm, respectively, of nitrite, potassium sorbate, and glycerol monolaurate, corresponding concentrations of 150, 5000, and 5000 ppm were required to inhibit aerobic growth (20 meq lactic acid added).*

*Sorbic acid, a 3:1 mixture of sorbic acid and glycerol monolaurate by weight, and butylated hydroxyanisole (BHA), however, did not show differential inhibitory effects toward aerobic and anaerobic growth of S. aureus and also were more effective inhibitors with addition of lactic acid. Sorbic acid suppressed staphylococcal growth in the model sausage system at 500 ppm, the mixture of sorbic acid and glycerol monolaurate at 750 ppm, and BHA at 10,000 ppm (20 meq lactic acid added).*

## INTRODUCTION

The growth of *Staphylococcus aureus* and production of enterotoxin in fermented sausages, particularly in Genoa and Italian dry salami, have caused numerous outbreaks of food poisoning (Center for Disease Control 1971a, 1971b, 1971c, 1975, 1979). Nitrite (and/or nitrate), the microbial inhibitor normally added to dry and semi-dry sausages, appears ineffective in controlling the growth of *S. aureus* under certain conditions.

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Nitrite inhibited the growth of *S. aureus* in culture media under anaerobic but not aerobic conditions (Castellani and Niven 1955; Lechowich *et al.* 1956; Buchanan and Solberg 1972). Nitrite became more effective as an inhibitor when pH decreased from 7.0 to 5.0 (Castellani and Niven 1955). In fermented sausages containing nitrite, the inner core (anaerobic portion) contained fewer staphylococci than the surface (aerobic portion) (Barber and Deibel 1972; Labots 1976; Lee *et al.* 1977). Anaerobic inhibition of staphylococcal growth by nitrite is so well established that the National Research Council's Committee on Food Protection criterion for evaluation of enterotoxin formation in fermented sausages consists of the enumeration of coagulase positive *S. aureus* only in the outer 6 to 7 mm portion (National Research Council 1975).

Rapid acidification of fermented sausages through use of active starter cultures is recommended as the means to inhibit the growth of staphylococci (National Research Council 1975). Because of the occasional failure of lactic acid bacteria (either natural flora or starter culture) in producing acid rapidly in sausages, an inhibitor of staphylococci should be present to prevent aerobic growth in *S. aureus*. In this study, compounds other than nitrite that could inhibit the growth of staphylococci in a model sausage system were examined.

## MATERIALS AND METHODS

A sterile agar-meat sausage system was developed for the investigation of the effect of various inhibitory compounds on the aerobic and anaerobic growth of *S. aureus*, since staphylococci are easily overgrown by natural meat flora.

### Preparation of *S. Aureus* Inoculum

Ten ml Tryptic Soy broth (Difco<sup>2</sup>) contained in a screw cap tube were inoculated with *S. aureus* 196E and incubated at 35°C for 24 h. One ml of a 1/100 dilution of the broth culture was added to the blended meat prior to addition of the tempered agar solution (see below). Initial cell count of the agar-meat sausages was approximately  $3 \times 10^3$  *S. aureus*/g.

### Preparation of Agar-Meat Sausages

One hundred gram quantities of ground pork shoulder (ground through a 3.2 mm plate) were placed in 200 ml beakers, covered with aluminum foil, and autoclaved at 15 psi for 20 min. When cool, the meat was placed in sterile one-liter stainless steel blender jars and blended for 10 s followed

<sup>2</sup> Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

by the addition of the *S. aureus* inoculum. To each jar was added 150 ml of a tempered (70°C) agar solution (autoclaved at 15 psi for 20 min) containing 7.5 g NaCl, 5 g agar, and 0.5 g glucose. The meat and agar solution was blended for 10 s under sterile nitrogen gas. The final concentration of NaCl, agar, and glucose was 3, 2, and 0.2%, respectively. The blended solution was poured immediately into sterilized 55 × 120 mm moisture-proof casings (Union Carbide, Chicago, IL; autoclaved in distilled water at 15 psi for 20 min). The agar-meat sausages were solidified in ice water and incubated at 35°C for 3 days in an upright position. The test compounds: butylated hydroxyanisole; potassium sorbate; glycerol monolaurate (Lauricidin™, distilled monoglyceride content ≥ 90%); sorbic acid; and Lauribic™ (a 3:1 w/w mixture of sorbic acid and glycerol monolaurate) were weighed as dry powders into sterile blender jars. Lauricidin™ and Lauribic™ were obtained from J. Kabara (Michigan State University). Lactic acid and sodium nitrite were dissolved in sterile distilled water. None of the additives were sterilized.

#### Enumeration of *S. aureus*

The ends were removed aseptically from each agar-meat sausage after 3 days incubation at 35°C, and sausages were cut in half. A 35 mm stainless steel tube, sterilized by flaming, was pushed through the center of each piece to give a central anaerobic portion (diameter, 33 mm) and an outer ring (aerobic portion). Fifty grams of each portion was placed in a sterile plastic bag with 200 ml of sterile 0.1% peptone (Difco) water added, and the mixture was blended for 2 min in a #400 Stomacher (Cooke Laboratory Products, Alexandria, VA). Appropriate dilutions of each mixture were plated on the surface of Tryptic Soy agar (Difco) plates with a Spiral Plater (Spiral Systems Marketing, Bethesda, MD). The plates were incubated at 35°C, and typical golden colonies were counted after 2 days.

#### Determination of pH

The pH at the surface of the agar-meat sausages was determined with an Owens-Illinois combination 2000 surface electrode attached to a Beckman pH meter (Model 76).

## RESULTS

Preliminary experiments indicated that the test compounds were more active as inhibitors of *S. aureus* growth in the agar-meat sausage

system when lactic acid was added. Addition of 0 to 20 meq lactic acid had little effect on the growth of staphylococci at 72 h. The pH of the agar-meat sausages changed from 5.7 with no added acid to 5.4 with 20 meq (Table 1). At higher concentrations of lactic acid, little or no growth of *S. aureus* occurred at 72 h; the pH dropped from 5.7 with no acid to 5.1 with 25 or 30 meq lactic acid. Lactic acid (20 meq) was added to produce acidic conditions in the agar meat sausage since that concentration did not inhibit *S. aureus* growth (Table 1). Sodium lactate added at the same molar concentration did not duplicate the observed effect of lactic acid.

Table 1. Effect of lactic acid on the aerobic and anaerobic growth of *S. aureus* after 72 h incubation at 35°C

| Concentration<br>of<br>Lactic Acid<br>(meq) | Sausage<br>pH | Log <sub>10</sub> Viable <i>S. aureus</i> /g <sup>a</sup> |                  |
|---------------------------------------------|---------------|-----------------------------------------------------------|------------------|
|                                             |               | Aerobic Growth                                            | Anaerobic Growth |
| 0                                           | 5.7           | 7.18                                                      | 7.04             |
| 10                                          | 5.3           | 7.64                                                      | 7.32             |
| 20                                          | 5.4           | 7.23                                                      | 7.28             |
| 25                                          | 5.1           | 4.00                                                      | 2.60             |
| 30                                          | 5.1           | 3.85                                                      | 2.48             |

<sup>a</sup>Initial inoculum of *S. aureus* was 3.43 (log<sub>10</sub> viable *S. aureus*/g)

In the absence of added lactic acid, none of the inhibitors completely prevented growth of *S. aureus* aerobically or anaerobically (Fig. 1A and 1B). Glycerol monolaurate (5000 ppm) and BHA (10,000 ppm) gave slight inhibition of growth aerobically; the other compounds had no effect (Fig. 1A). Under anaerobic conditions, potassium sorbate (5,000 ppm), NaNO<sub>2</sub> (100 and 200 ppm), glycerol monolaurate (5000 ppm), and BHA (10,000 ppm) gave reduced growth but did not completely suppress staphylococcal growth (Fig. 1B).

The data presented in Figs. 2A and 2B indicate that addition of 20 meq lactic acid, while not inhibitory in itself (Table 1), enhanced the growth suppressive action of all of the test compounds (compare to data presented in Fig. 1A and 1B in which lactic acid was not added). Sorbic acid, Lauribic <sup>TM</sup>, and BHA behaved similarly, in that aerobic and anaerobic growth of *S. aureus* were prevented by approximately the same concentration of inhibitor. At a level of 500 ppm, sorbic acid inhibited both aerobic and anaerobic growth equally well (Fig. 2A and

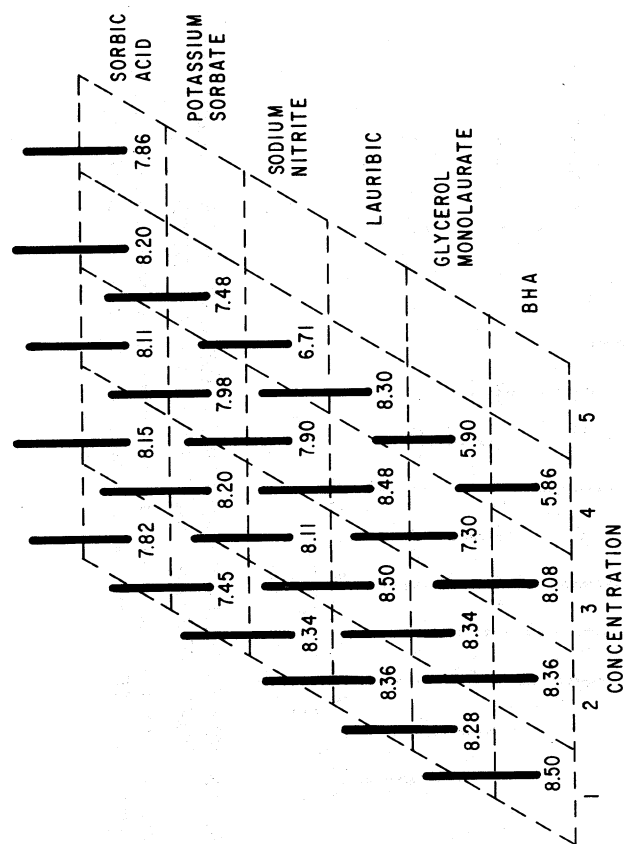


FIG. 1A. THE EFFECT OF VARIOUS INHIBITORS ON THE AEROBIC GROWTH OF *S. AUREUS* (35°C, 72h, NO ADDED ACID)

Numbers beneath bars represent  $\log_{10}$  viable *S. aureus*/g. Initial inoculum ranged from 3.20–3.66 ( $\log_{10}$  viable *S. aureus*/g). Concentration in ppm (1 = 0 ppm): sorbic acid – 0, 250, 500, 750, 1000; potassium sorbate – 0, 1250, 2500, 5000; sodium nitrite – 0, 50, 100, 200; Lauricic™ – 0, 500, 750, 1000; glycerol monolaurate – 0, 1250, 2500, 5000; BHA – 0, 2500, 5000, 10,000.

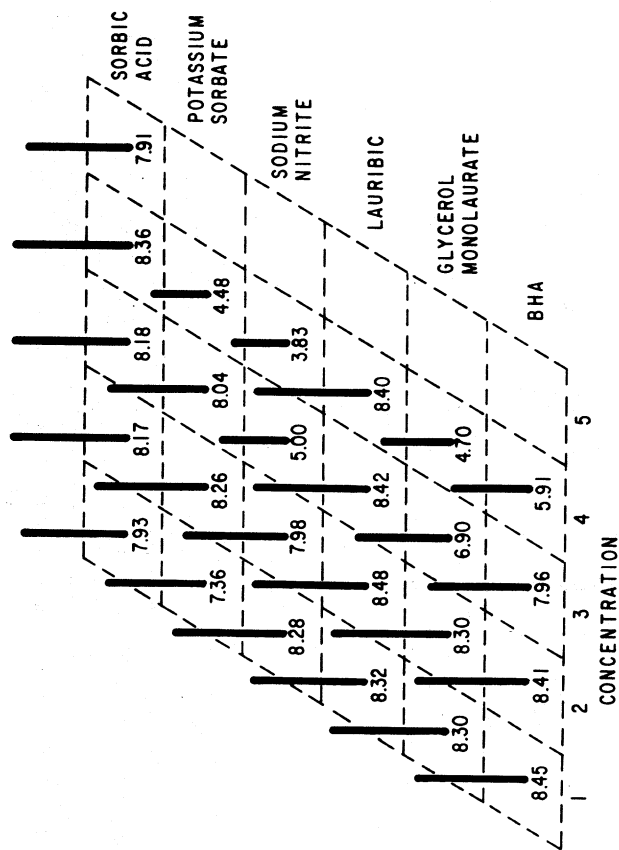


FIG. 1B. THE EFFECT OF VARIOUS INHIBITORS ON THE ANAEROBIC GROWTH OF *S. AUREUS* (35°C, 72 h, NO ADDED ACID)

Numbers beneath bars represent  $\log_{10}$  viable *S. aureus*/g. Initial inoculum ranged from 3.20 – 3.66 ( $\log_{10}$  viable *S. aureus*/g). Concentrations given in Fig. 1A.

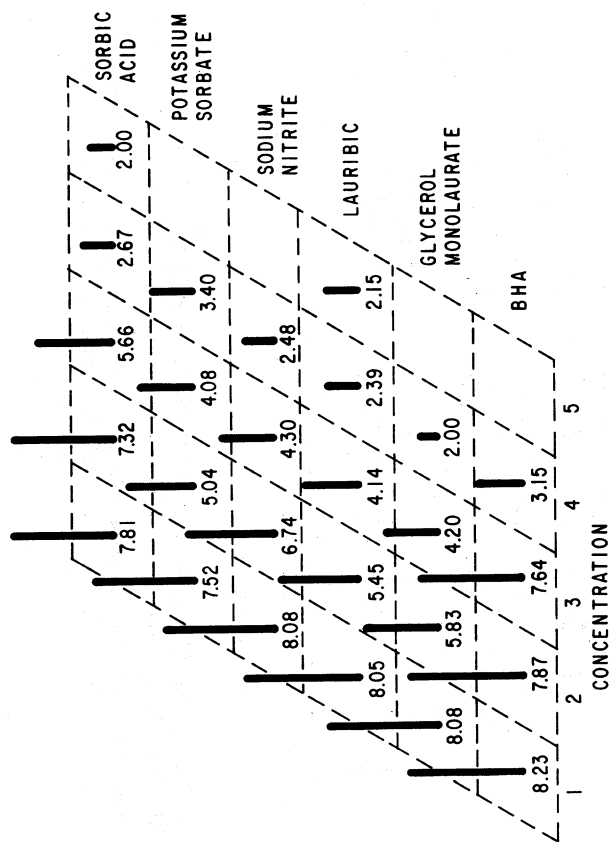


FIG. 2A. THE EFFECT OF VARIOUS INHIBITORS ON THE AEROBIC GROWTH OF *S. AUREUS* (35°C, 72 h, 20 meq ADDED LACTIC ACID)

Numbers beneath bars represent  $\log_{10}$  viable *S. aureus*/g. Initial inoculum ranged from 3.20 – 3.66 ( $\log_{10}$  viable *S. aureus*/g). Concentration in ppm (1 = 0 ppm): sorbic acid – 0, 125, 250, 500, 750; potassium sorbate – 0, 1250, 2500, 5000; sodium nitrite – 0, 50, 100, 200; Lauric<sup>TM</sup> – 0, 250, 500, 750, 1000; glycerol monolaurate – 0, 1250, 2500, 5000; BHA – 0, 2500, 5000, 10,000

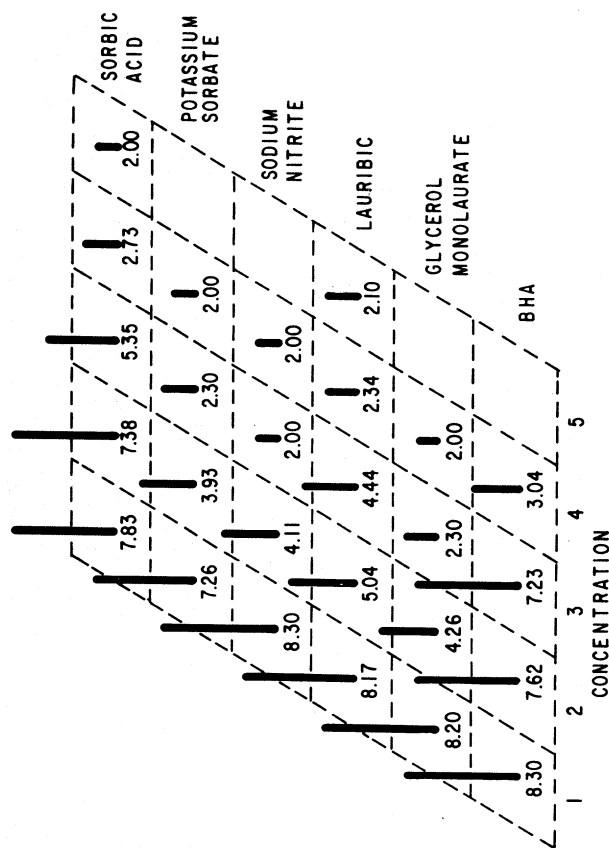


FIG. 2B. THE EFFECT OF VARIOUS INHIBITORS ON THE ANAEROBIC GROWTH OF *S. AUREUS* (35°C, 72 h, 20 meq ADDED LACTIC ACID) Numbers beneath bars represent log<sub>10</sub> viable *S. aureus*/g. Initial inoculum ranged from 3.20 — 3.66 (log<sub>10</sub> viable *S. aureus*/g). Concentrations given in Fig. 2A



2B). Lauribic™ at 750 ppm and BHA at 10,000 ppm were effective in preventing growth aerobically and anaerobically (Fig. 2A and 2B).

The aerobic growth of staphylococci was inhibited by 5000 ppm potassium sorbate or glycerol monolaurate (Fig. 2A) but only 2500 ppm of either compound was necessary to inhibit anaerobic growth (Fig. 2B). Sodium nitrite prevented growth aerobically at 200 ppm but only 100 ppm was needed under anaerobic conditions (Fig. 2A and 2B). Thus, potassium sorbate, glycerol monolaurate, and sodium nitrite behaved differently from the other inhibitors in that anaerobic growth of *S. aureus* was more susceptible than aerobic growth to the toxic agents.

The pH values of the sausages containing varying amounts of inhibitors are presented in Table 2. Of the 24 combinations of inhibitor with or without added lactic acid under aerobic or anaerobic conditions, only 4 showed statistically significant correlation ( $P < 0.05$ ) between pH and extent of inhibition of *S. aureus* growth: (1) nitrite, no added acid, aerobic; (2) BHA, no added acid, aerobic; (3) potassium sorbate, no added acid, anaerobic; and (4) glycerol monolaurate, acid added, anaerobic. Thus, for most combinations, there was no relationship between pH and the extent of inhibition by the test compound.

## DISCUSSION

Microbial inhibitors act by various mechanisms to limit cell division and growth. Those inhibitors used in foods for human consumption must be Generally Regarded As Safe (GRAS) at the concentrations normally employed for microbial inhibition. At the present time, all of the test substances used in this study are GRAS and were selected as examples of different mechanisms for microbial inhibitions.

Lactic acid ( $pK_a$ , 3.85) is an acidulant inhibitor normally present in many foods produced in minor quantities from metabolic reactions. Lactic acid is produced in larger quantities in certain acid type foods by microbial fermentation. The low pH and acidic environment inhibit most bacterial growth. Nitrite, the salt of nitrous acid ( $pK_a$ , 3.38), is added to cured meats to inhibit the outgrowth of *Clostridium botulinum* spores by an as yet undefined mechanism. Nitrite also inhibits other microorganisms such as staphylococci by inactivating sulfhydryl-containing enzymes (Buchanan and Solberg 1972). Sorbic acid (2,4-hexadienoic acid;  $pK_a$ , 4.8) and its salt, potassium sorbate, are widely used for inhibiting fungal growth (Chichester and Tanner 1973) and appear to act on the cell membrane to inhibit substrate uptake (Freese *et al.* 1973). Sorbic acid is a lipophilic unsaturated aliphatic acid whose inhibitory activ-

Table 2. pH of sausages after 72 h incubation at 35°C

| Compound                        | pH of Sausages at the<br>Following Concentrations of Inhibitor |     |     |     |     |
|---------------------------------|----------------------------------------------------------------|-----|-----|-----|-----|
|                                 | 1                                                              | 2   | 3   | 4   | 5   |
| <i>Lactic acid not added</i>    |                                                                |     |     |     |     |
| Sorbic acid <sup>a</sup>        | 5.5                                                            | 5.4 | 5.4 | 5.3 | 5.5 |
| Potassium sorbate               | 5.6                                                            | 5.5 | 5.5 | 6.1 |     |
| Sodium nitrite                  | 5.6                                                            | 5.6 | 5.6 | 5.9 |     |
| Lauribic <sup>TM</sup>          | 5.7                                                            | 5.4 | 5.3 | 5.3 |     |
| Glycerol monolaurate            | 5.6                                                            | 5.6 | 6.1 | 6.0 |     |
| BHA                             | 5.6                                                            | 5.6 | 5.5 | 6.1 |     |
| <i>20 meq lactic acid added</i> |                                                                |     |     |     |     |
| Sorbic acid <sup>b</sup>        | 5.3                                                            | 5.5 | 5.4 | 5.3 | 5.4 |
| Potassium sorbate               | 5.5                                                            | 5.6 | 5.6 | 5.7 |     |
| Sodium nitrite                  | 5.2                                                            | 5.5 | 5.5 | 5.4 |     |
| Lauribic <sup>TM</sup>          | 5.2                                                            | 5.4 | 5.4 | 5.3 | 5.3 |
| Glycerol monolaurate            | 5.3                                                            | 5.5 | 5.5 | 5.6 |     |
| BHA                             | 5.2                                                            | 5.5 | 5.5 | 5.5 |     |

<sup>a</sup>Concentration in ppm (1=0 ppm): sorbic acid — 0, 250, 500, 750, 1000; potassium sorbate — 0, 1250, 2500, 5000; sodium nitrite — 0, 50, 100, 200; Lauribic<sup>TM</sup> — 0, 500, 750, 1000; glycerol monolaurate — 0, 1250, 2500, 5000; BHA — 0, 2500, 5000, 10,000

<sup>b</sup>Concentration in ppm (1=0 ppm): sorbic acid — 0, 125, 250, 500, 750; potassium sorbate — 0, 1250, 2500, 5000; sodium nitrite — 0, 50, 100, 200; Lauribic<sup>TM</sup> — 0, 250, 500, 750, 1000; glycerol monolaurate — 0, 1250, 2500, 5000; BHA — 0, 2500, 5000, 10,000

ity is strongly pH dependent with increased inhibitory activity at lower pH. BHA is a sterically hindered lipophilic phenol which is normally added to foods as an antioxidant. It has antibacterial activity (Ayaz *et al.* 1980), possibly from the phenolic group. Glycerol monolaurate, a monoglyceride of lauric acid (dodecanoic acid;  $pK_a$ , 4.8), is an amphiphilic surface-active agent and is often added to foods as an emulsifier; its antimicrobial properties have been reported (Kabara *et al.* 1977). Lauribic<sup>TM</sup> is a proprietary mixture of sorbic acid and glycerol monolaurate used as a microbial inhibitor (Kabara 1980). The mechanism of action of Lauribic<sup>TM</sup> is unknown but might be expected to act by a surface-active mechanism on the microbial cell membrane to disrupt substrate utilization. The antimicrobial action of Lauribic<sup>TM</sup> would be expected to be pH dependent due to its high content of sorbic acid.

All the test compounds were more effective as inhibitors of the growth of *S. aureus* when lactic acid was added; an equimolar level of sodium

lactate did not substitute for the lactic acid. In general, there was no relationship between inhibition of growth by the test compounds and the pH of the sausages.

The data obtained in this study indicated that  $\text{NaNO}_2$ , potassium sorbate, and glycerol monolaurate were similar in that they were more effective inhibitors of *S. aureus* growth under anaerobic conditions, as well as more effective when lactic acid was added. Approximately twice as much inhibitor was required to limit aerobic than anaerobic growth.

Tompkin *et al.* (1974) indicated that potassium sorbate was not effective in inhibiting growth of *S. aureus* inoculated on the surface of breakfast sausages incubated at 27°C. In vacuum-packed sliced bacon incubated at 27°C, potassium sorbate suppressed the growth of *S. aureus* for 14 days (Pierson *et al.* 1979). Thus, the work of Tompkin *et al.* (1974) and Pierson *et al.* (1979) suggest that potassium sorbate is more potent as an inhibitor of anaerobic staphylococcal growth.

The proposal has been made that glycerol monolaurate be made GRAS as an antimicrobial additive in foods (Federal Register 1979). Glycerol monolaurate has been shown to be active against staphylococci in bacteriological media at a minimal inhibitory concentration (MIC) of approximately 26 ppm (Kabara *et al.* 1972). The MIC against *S. aureus* decreased as the pH decreased from 7 to 5 (Kabara *et al.* 1977). In the agar-meat sausages, the levels of glycerol monolaurate that prevented growth of *S. aureus* ranged from 2500 ppm (anaerobic) to 5000 ppm (aerobic) indicating that the inhibitor was much less active in a food system.

The three remaining compounds that were tested — sorbic acid, Lauribic<sup>TM</sup> and BHA — behaved similarly in that both aerobic and anaerobic growth of *S. aureus* were suppressed by the same concentration of inhibitor and that lactic acid was needed for effectiveness.

Lauribic<sup>TM</sup>, a proprietary combination of sorbic acid and Lauricidin<sup>TM</sup> (glycerol monolaurate, 90%) had a MIC of 62.5 ppm against *S. aureus* in bacteriological media (pH 5.5) in contrast to sorbic acid which had a MIC of 5000 ppm under identical conditions (Kabara 1980). However, in the conditions used in the present study, Lauribic<sup>TM</sup> and sorbic acid had similar inhibitory abilities against *S. aureus*.

In bacteriological media, the growth of *S. aureus* was inhibited by BHA at 150 to 400 ppm (Chang and Branen 1975; Shih and Harris 1977; Stern *et al.* 1979; Ayaz *et al.* 1980). However, the presence of lipids decreased the effectiveness of BHA as a microbial inhibitor (Robach *et al.* 1977; Klindworth *et al.* 1979). The presence of lipid in the agar-meat sausage system probably accounts for the high level (approximately 10,000 ppm) of BHA necessary to inhibit the growth of *S. aureus*.

All of the test compounds inhibited the growth of *S. aureus*. The most effective inhibitors of staphylococcal growth in the agar-meat sausage system were nitrite and sorbic acid (including Lauribic™ which contains a large proportion of sorbic acid). Sorbic acid is less toxic than sodium nitrite; the oral LD<sub>50</sub> for rats is 7360 mg/kg for sorbic acid and 180 mg/kg for sodium nitrite (Merck Index 1976). Thus, the safety factor for sorbic acid over nitrite is approximately 40-fold. This safety factor and the ability to inhibit both aerobic and anaerobic growth of *S. aureus* gave sorbic acid advantages over nitrite. Sorbic acid would be useful in fermented sausage manufacture for the control of staphylococcal growth because lactic acid bacteria, including *Lactobacillus plantarum* (which is used in meat starter cultures), are resistant to sorbic acid (Emard and Vaughn 1952).

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